Electronic Supplementary Material (ESI) for ChemComm.
This journal is © The Royal Society of Chemistry 2017

Supporting information

Incorporation studies of clickable ceramides in Jurkat cell plasma membranes

T. Walter[a†], J. Schlegel[b†], A. Burgert[b], A. Kurz[b], J. Seibel[a] and M. Sauer[b]

[a]Institute for Organic Chemistry, Julius-Maximilians University Würzburg, Am Hubland C1, 97074 Würzburg, Germany.
Fax: +49 (0) 931 31 84625; Tel: +49 (0) 931 31 85326
Email: seibel@chemie.uni-wuerzburg.de.
[b]Department of Biotechnology and Biophysics, Julius-Maximilians-University Würzburg, 97074 Würzburg, Germany
Email: m.sauer@ uni-wuerzburg.de.
[†]The authors contributed equally to this work.

Experimental Section

Cell Culture. Jurkat T cells E6-1 (Cell Lines Service) were cultured in RPMI-1640 (Sigma) supplemented with 10 % FCS, 2 mM glutamine, 2 mM sodium pyruvate, 2 mM non-essential amino acids and 100 U/ml penicillin/streptomycin at 37 °C and 5 % CO_2. Cells were grown up to a density of ~ 1x10^6 cells/ml before splitting.

Labeling and confocal microscopy imaging. For imaging Jurkat cells were seeded into poly-D-lysine coated 8 well chamber slides (Lab-Tek II, Nunc; Thermo Fischer Scientific) at a concentration of 1x10^5 cells/well. After 90 min at 37 °C cells were incubated with 25 µM azido-functionalized ceramides (CerC6*α*, CerC6*ω*, CerC16*α* or CerC16*ω*) or preclicked ceramides (CerC6*α*pre, CerC6*ω*pre, CerC16*α*pre or CerC16*ω*pre) for 30 min at 37 °C. For click reactions cells were washed once with HBSS before they were labeled with 25 µM azadibenzocyclooctyne-Cy5 (DBCO-Cy5, Sigma-Aldrich) for 7 min at RT via the so-called strain-promoted azide-alkyne cycloaddition.[1] After washing cells three times with HBSS living cells were measured instantly. Confocal Laser Scanning Microscopy (CLSM) imaging was performed using a LSM700 (Zeiss, Germany) equipped with a Plan-Apochromat 63 x 1.4 oil objective. Cy5 fluorophores were excited with a 639 nm solid state laser. Images were processed using LSM software Zen system 2012 and Fiji.[2] Tris(2-carboxyethyl)phosphine hydrochloride solution (TCEP, pH 7.0; Sigma Aldrich) was diluted to 50 mM in HBSS and used for phosphine quenching of DBCO-Cy5[3] Jurkat cells were seeded in 200 µl HBSS and labelled as described above

whereas all parameters for time series measurements were kept constant (10 % laser intensity, pixel dwell time 1.87 µsec). Cells were imaged for 16 cycles with an interval of 10 seconds and 200 µl TCEP solution was added after 20 seconds to obtain a final working concentration of 25 mM.

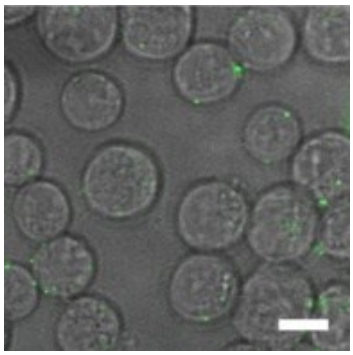
Anisotropy measurements. For anisotropy measurements 1x10^5 Jurkat cells were fed with 25 µM azido-functionalized or preclicked ceramides in 1,5 ml microcentrifuge tubes for 30 min at 37 °C. Cells were centrifuged at 300 x g for 5 min and washed with 500 µl HBSS. Subsequently, cells were incubated with 25 µM DBCO-Cy5 for 7 min at RT before they were washed two times with HBSS. Anisotropy measurements were performed using a spectrofluorometer (FP-6500, Jasco GmbH, Gross-Umstadt, Germany). In both, the emission and excitation path a polarizer (Thorlabs, LPVISE 100-A) was placed. Steady-state-anisotropy r was calculated by

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}}$$

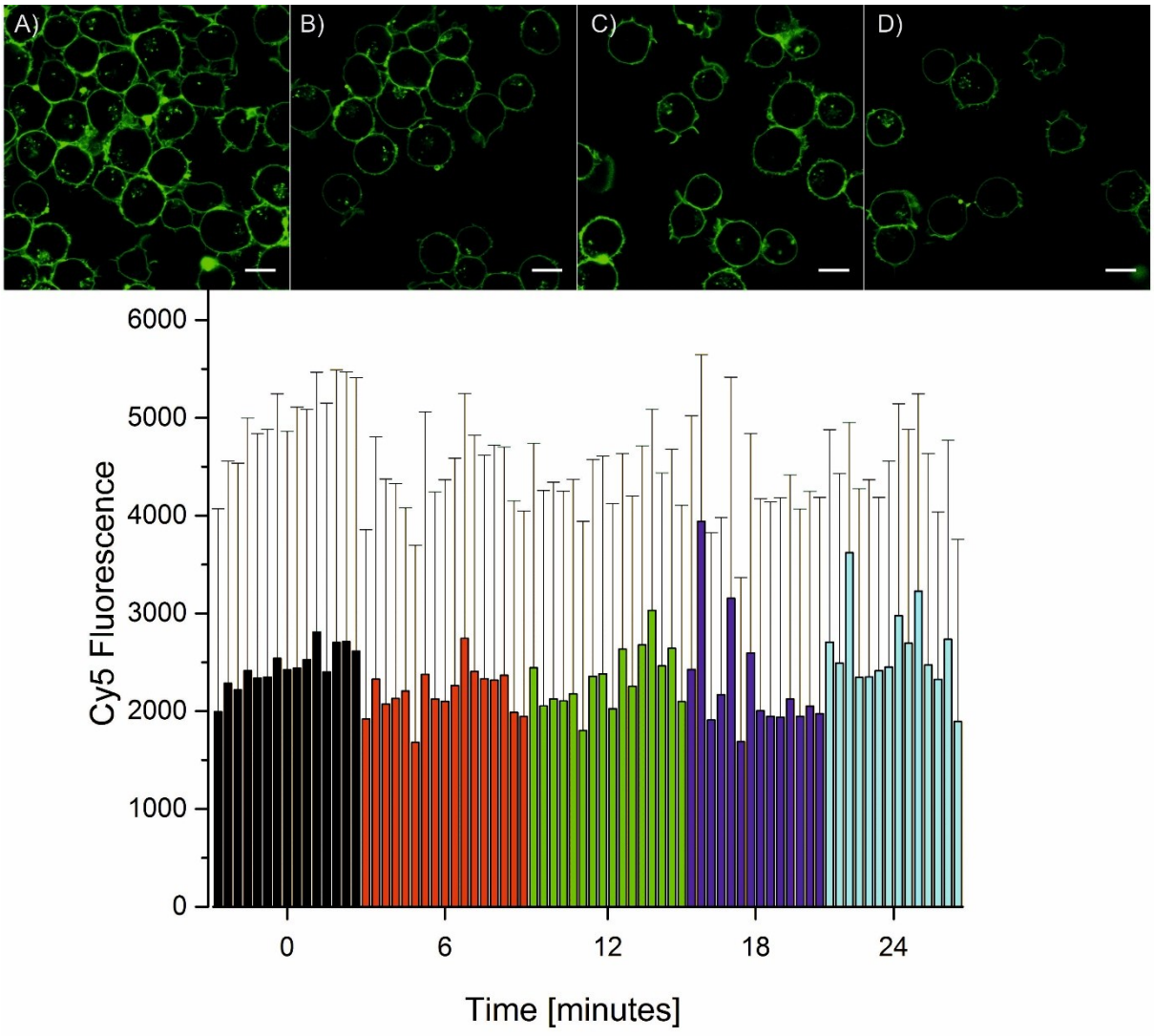
using

$$G = \frac{I_{HV}}{I_{HH}}$$

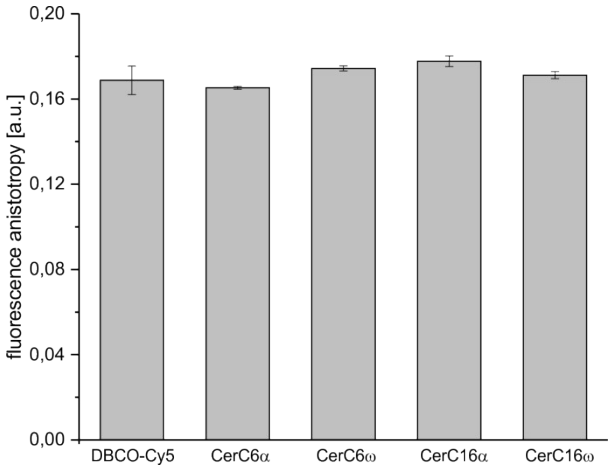
as a device correction factor for different sensitivities of the detection system concerning polarized light.[4] The indices V and H stand for vertically and horizontally oriented polarizers, the first index refers to the polarizer in the excitation path, the second in the detection path. The four different polarizer settings were measured consecutively 5 times for each sample. Excitation wavelength was set to 640 nm, detected wavelength to 670 nm. Bandwidth of these wavelengths had to be varied between 3-10 nm as well as the sensitivity of the detector to obtain a signal of the weakest polarizer setting (I_{HH}) in the range of 100 - 500 counts.



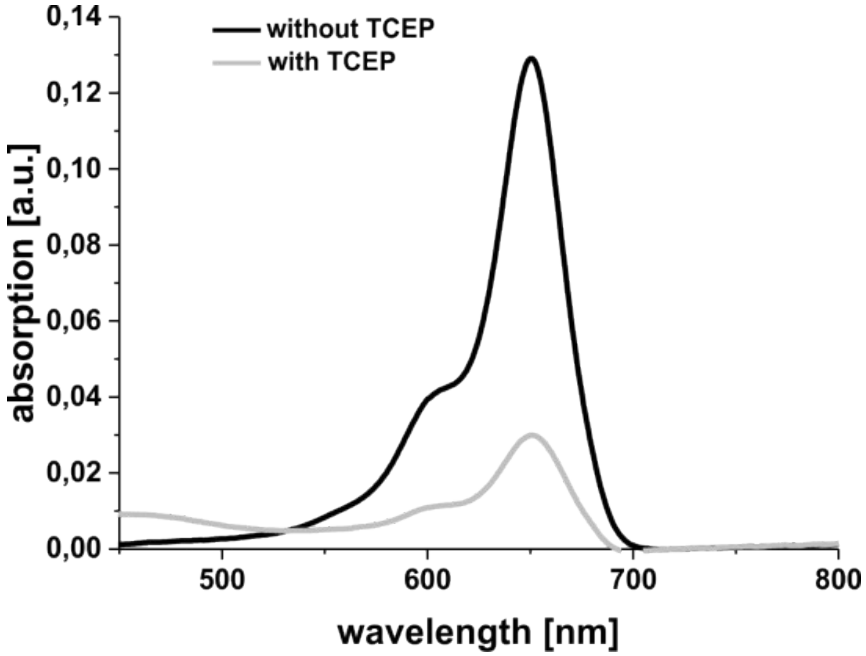
Supplementary Figure S1. LSM image of an untreated Jurkat cell after addition of 25 µM DBCO-Cy5 for 7 min. Scale bar, 10 µm.



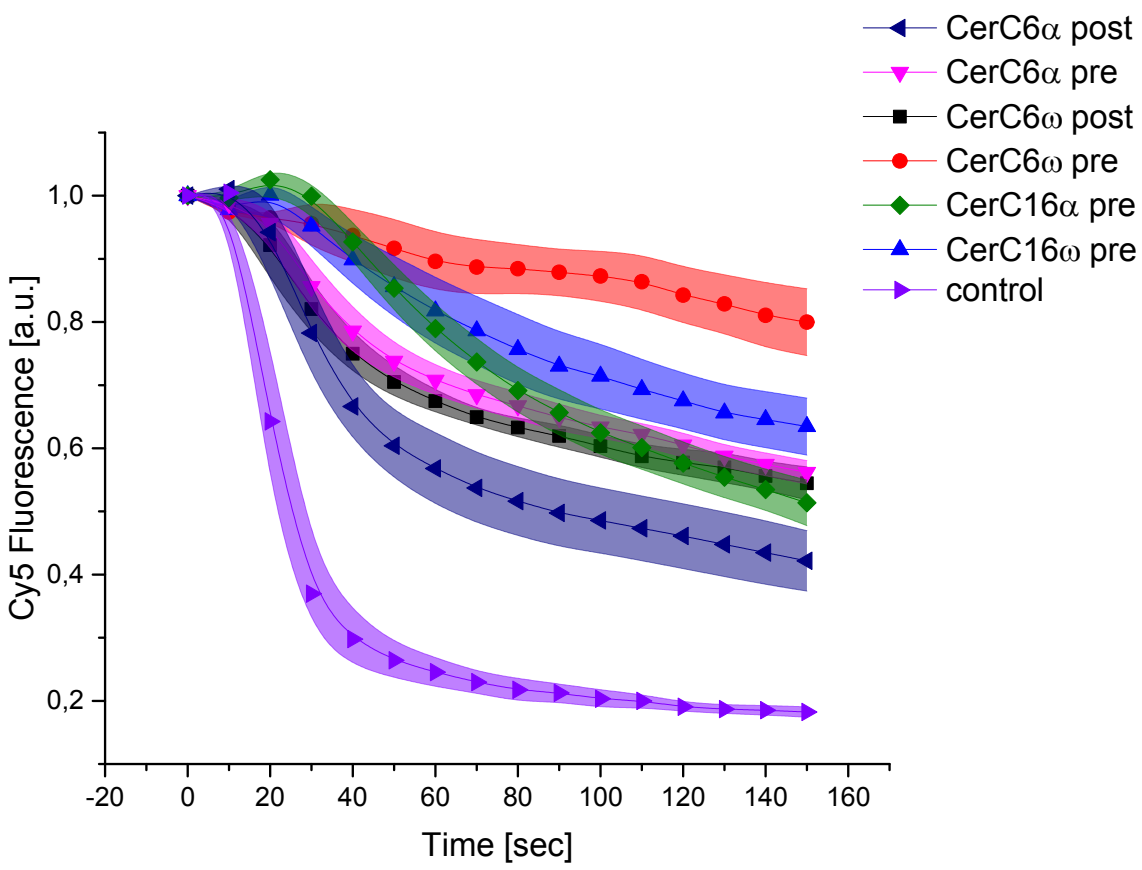
Supplementary Figure S2. LSM images of ceramide labelled Jurkat cells. Cells were attached onto poly-D-lysine coated glass surfaces and incubated for 30 minutes with 25 µM CerC6ω. Cells were thoroughly washed with HBSS and labelled with 25 µM DBCO-Cy5 after 0 min (A) 12 min (B) 18 min (C), and 24 min (D) at room temperature to follow internalization kinetics of non-labelled ceramides. Scale bar, 10 µm. Quantification of fluorescence intensities after different times demonstrates that the ceramide concentrations in the plasma membrane remains constant during the first 24 min after labelling.



Supplementary Figure S3. Steady-state fluorescence anisotropy values measured for DBCO-Cy5 and preclicked azido-functionalized ceramides in HBSS are identical. Data represent mean values with standard errors of two independent experiments with five measurements each.



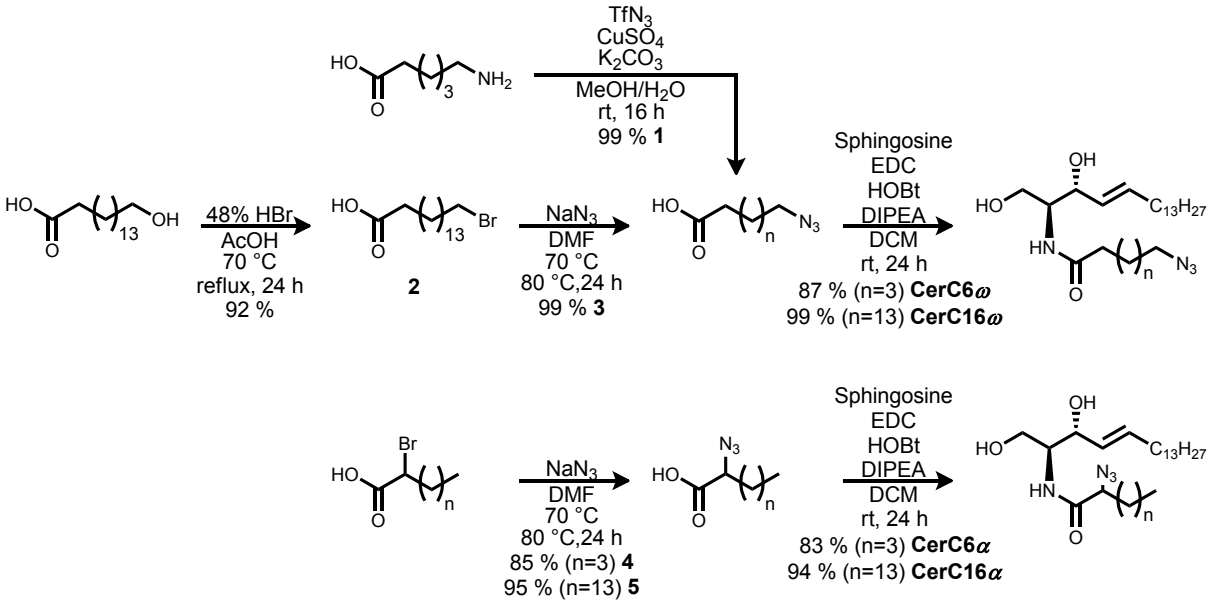
Supplementary Figure S4. Fluorescence quenching of DBCO-Cy5 by phosphine tris(2-carboxyethyl)phosphine (TCEP). Absorption spectra of DBCO-Cy5 in the absence and presence of 25 mM TCEP. The initial absorbance (black line) decreases upon addition of TCEP (gray line). TCEP reacts with Cy5 spontaneously to form a non-fluorescent adduct by conjugation to the polymethine bridge of the cyanine dye. The absorption spectra were recorded with a spectrophotometer (JASCO V-650).



Supplementary Figure S5. Live-cell fluorescence quenching of postclicked and preclicked ceramides in Jurkat cells by TCEP. TCEP was added to a final concentration of 25 mM at the beginning of the experiment (t = 0 s) and the resulting change in fluorescence intensity of cells was followed by confocal laser scanning microscopy (LSM). The control experiment was performed by adding DBCO-Cy5 to untreated Jurkat cells.

Chemical synthesis

General: Sphingosine was purchased from TCI (TCI Chemicals, Eschborn, Germany). Other commercially available reagents were used as received (Acros, Alfa Aesar, Merck, Sigma, TCI). Reactions were monitored by TLC analysis with the use of silica gel coated plates (0.2 mm thickness). The detection was achieved by following staining solutions: a) 0.60 g ninhydrin, 200 ml *n*-butanol, 6.00 ml glacial acid b) 1.50 g $KMnO_4$, 10.0 g K_2CO_3, 100 mg NaOH, 200 ml H_2O c) bromocresol green 0.08 g, 200 ml EtOH, addition of 0.1 M NaOH aq. till until the color of the solution remains blue. Azides were stained with solution a after a prior reduction with a 10 % solution of PPh_3 in toluol. Liquid column chromatography purification was performed using silica gel 60 (40–63 µm mesh). The NMR spectra were recorded with a BRUKER AVANCE 400 FT-NMR spectrometer at 25 °C. Chemical shifts are given in ppm (δ) relative to tetramethylsilane as internal standard. Mass spectrometry (MS) was performed on a BRUKER Daltonics autoflex II (electrospray ionization, ESI) instrument.



General procedere A acylation of sphingosine

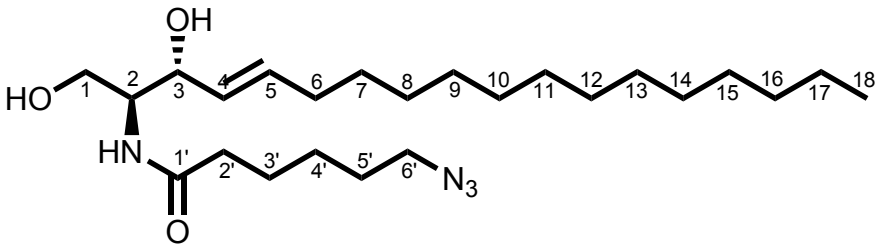
Azido-sphingosines were prepared by adding a solution of azide modified fatty acid (1.00 eq) in CH_2Cl_2 (60 ml per mmol sphingosine) dropwise to a mixture of sphingosine (1.00 eq.), HOBt (1.20 eq.), EDC (1.20 eq.) and DIPEA (1.80 eq.) in dry CH_2Cl_2 (60 ml per mmol sphingosine)

within 2 h at 0°C. followed by an 18 h stirring under nitrogen atmosphere. The solvent was removed and the residue was purified via column chromatography (CH_2Cl_2/MeOH 100:1).

General procedere B azidification of bromocarboxylic acids

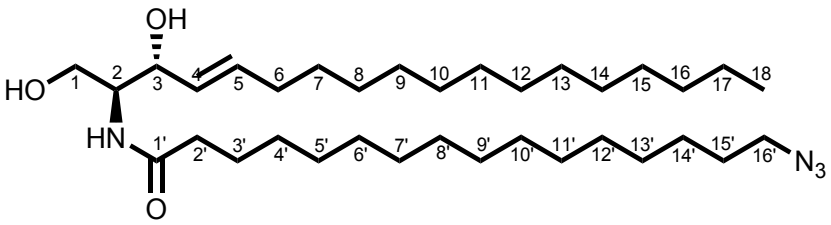
Bromocarboxylic acid (1.00 eq) and NaN_3 (2.00 eq) was stirred in dmf (10 ml per mmol acid) at 80 °C over night. The solvent was removed under reduced pressure and the redue solved in 0.5 M HCl (15 ml per mmol acid) an extraced three times with ethylacetate (10 ml per mmol acid). The organic layers were combined ant the solvents removed under reduced pressure. The residue was purified via column chromatography (cyclohexane/ethyl acetate) to yield the coresponding azidified carboxylic acid.

ω-Azido-C6-ceramide **CerC6*ω***



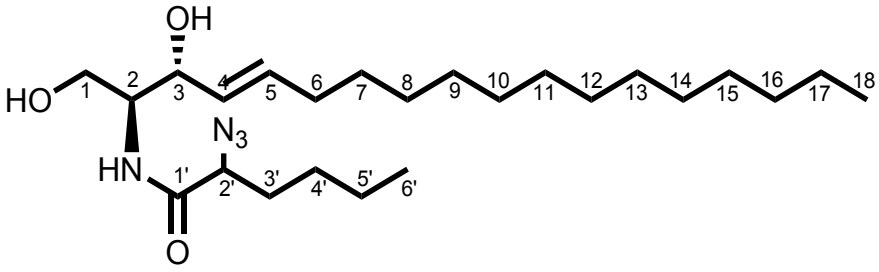
General procedure A gave **CerC6*ω*** as colorless solid. Yield 87 %. R_f: 0.72 (CH_2Cl_2/MeOH 10:1). ^{1}-NMR: (400 MHz, $CDCl_3$): *δ* = 6.32 (d, ^3J = 7.6 Hz, 1H; N*H*), 5.78 (dddd, ^3J = 15.3, 6.9, 6.9, ^4J = 1.2 Hz, 1H; *H*-5), 5.52 (dddd, ^3J = 15.4, 6.4, ^4J = 1.3, 1.3 Hz, 1H; *H*-4), 4.35-4.29 (m, 1H; *H*-3), 3.95 (dd, ^2J = 11.1, ^3J = 3.7, 1H; *H*-1a), 3.91 (dddd, ^3J =7.4, 3.7, 3.7, 3.7 Hz, 1H, *H*-2), 3.74-3.66 (m, 1H; *H*-1b), 3.28 (dd, ^3J = 6.8, 6.8 Hz, 2H; *H*-6'), 2.87 (s, 2H; O*H*), 2.25 (dd, ^3J = 7.5, 7.5 Hz, 2H; *H*-2'), 2.05 (dd, ^3J = 14.2, 6.9 Hz 2H; *H*-6), 1.73-1.66 (m, 2H; *H*-3'), 1.73-1.58 (m, 2H, *H*-5'), 1.15-1.25 (m, 24H; *H*-7 to *H*-17, *H*-4'), 0.87 (dd, ^3J = 6.9 Hz, 3H; *H*-18) ppm. ^{13}*C*-NMR: (100 MHz, $CDCl_3$): *δ* =173.5 (*C*-1'), 134.4 (*C*-5), 128.8 (*C*-6), 74.8 (*C*-3), 62.5 (*C*-1), 54.5 (*C*-2), 51.4 (*C*-6'), 36.6 (*C*-2'), 32.4 (*C*-6), 32.1, 29.8, 29.8, 29.8, 29.6, 29.5, 29.4, 29.3 (10C, *C*-alkyl), 28.7 (*C*-5'), 26.4 (*C*-4'), 25.3 (*C*-3'), 22.8 (1C, *C*-alkyl), 14.3 (*C*-18) ppm. HRMS-ESI (+), *m/z*: 461.34614 [M+Na$^+$] calcd. for $C_{24}H_{46}N_4NaO_3^+$: 461.34621.

ω-Azido-C16-ceramide **CerC16*ω***



General procedure A gave **CerC16*ω*** as colorless solid. Yield 99 %. R_f: 0.50 (CH_2Cl_2/MeOH 10:1). ^{1}H-NMR: (400 MHz, $CDCl_3$): *δ* = 6.25 (d, ^3J = 7.4 Hz, 1H; N*H*), 5.78 (dddd, ^3J = 15.2, 6.8, 6.8, ^4J = 1.1 Hz, 1H; *H*-5), 5.52 (dddd, ^3J = 15.4, 6.4, ^4J = 1.3, 1.3 Hz, 1H; *H*-4), 4.35-4.29 (m, 1H; *H*-3), 3.98-3.95 (m, 1H, *H*-1a), 3.90 (ddd, ^3J = 7.4, 7.4, 3.7 Hz, 1H, *H*-2), 3.70 (ddd, ^2J = 10.7, ^3J = 7.0, 3.4, 1H *H*-1b), 3.25 (t ^3J = 6.98, 1H; *H*-16'), 2.76-2.70 (m, 2H, O*H*), 2.24 (dd, ^3J = 7.9, 7.3 Hz, 2H; *H*-2'), 2.05 (ddd, ^3J = 7.1, 7.1, 7.1 Hz 2H; *H*-6), 1.67-1.56 (m, 4H; *H*-3‘, *H*-15'), 1.15-1.25 (m, 44H; *H*-7 to *H*-17, *H*-4'-H14'), 0.88 (dd, ^3J = 6.9, 6.9 Hz, 3H; *H*-18) ppm. ^{13}*C*-NMR: (100 MHz, $CDCl_3$): *δ* = 174.0 (*C*-1'), 134.5 (*C*-5), 128.9 (*C*-6), 74.9 (*C*-3), 62.7 (*C*-1), 54.6 (*C*-2), 51.6 (*C*-16'), 37.0 (*C*-2'), 32.4 (*C*-6), 32.1, 29.8, 29.8, 29.8, 29.7, 29.6, 29.6, 29.5, 29.4, 29.4, 29.3, 29.3 (20C, *C*-alkyl), 29.0 (*C*-15'), 26.9 (1C, *C*-alkyl), 25.9 (*C*-3'), 22.8 (1C, *C*-alkyl), 14.3 (*C*-18) ppm HRMS-ESI (+), *m/z*: 579.51988 [M+H$^+$] calcd. for $C_{34}H_{67}N_4O_3^+$: 579.52077.

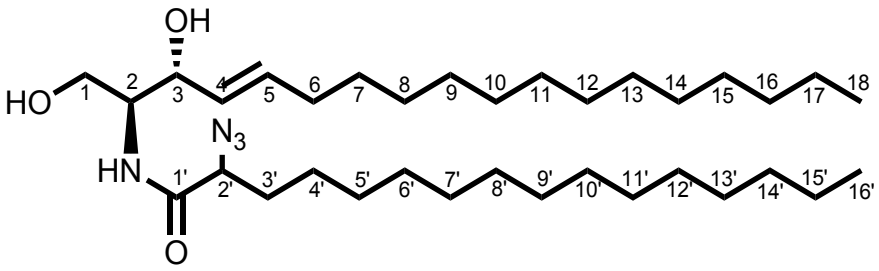
α-Azido-C6-ceramide **CerC6*α***



General procedure A gave **CerC6*α*** as colorless solid. Yield 83 %. R_f: 0.35 (CH_2Cl_2/MeOH 25:1). ^{1}H-NMR: (400 MHz, $CDCl_3$): *δ* = 7.02 (d, ^3J = 8.1 Hz, 2H; N*H*), 5.79 (dddd, ^3J = 15.3, 6.9, 6.9 ^4J = 1.1 Hz, 2H; *H*-5) respectively 5.79 (dddd, ^3J = 15.4, 6.8, 6.8 ^4J = 1.1 Hz, 2H; *H*-5), 5.51 (dddd, ^3J = 15.4, 6.4,^4J = 1.5, 1.5 Hz, 1H; *H*-4) respectively 5.50 (dddd, ^3J = 15.4, 6.4, ^4J = 1.5, 1.5 Hz, 1H; *H*-4), 4.32 (dd, ^3J = 5.3, 5.3 Hz 1H; H-3) respectively 4.28 (dd, ^3J = 5.3, 5.3 Hz 1H; H-3), 4.00-3.94 (m, 4H, H-1a, *H*-2'), 3.88 (dddd, ^3J = 9.0, 4.1, 3.9, 3.9 Hz; 1H, *H*-2) respectively 3.87 (dddd, ^3J = 8.2, 4.0, 4.0, 4.0 Hz; 1H, *H*-2), 3.71 (dd, ^3J = 10.7, 3.2 Hz, 1H, *H*-1), respectively 3.68 (dd, ^3J = 10.8, 3.2 Hz, 1H, *H*-1), 3.00-2.70 (m, 4H, *C*-1-O*H*, *C*-2-O*H*), 2.10-2.00 (m, 4H, *H*-6), 1.98-1.88

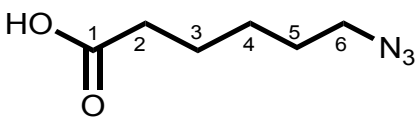
(m, 2H, *H*-3‘a), 1.83 (m, 2H, *H*-3'b), 1.47-1.19 (m, 52 Hs, *H*-alkyl), 0.92 (t, 7.2 Hz, 6H, *H*-6'), 0.87 (t, 6.9 Hz, 6H, *H*-18) ppm; ^{13}*C*-NMR: (100 MHz, $CDCl_3$): δ = 170,1 (2C, *C*-1'), 134,9 (2C, *C*-5), 128,7 (1C, *C*-4), 128,6 (1C, *C*-4), 74,4 (1C, *C*-3), 74,3 (1C, *C*-3), 64,5 (2C, *C*-2'), 62.4 (1C, *C*-1), 62.3 (1 C, *C*-1), 54.7 (1C, *C*-2), 54.6 (1C, *C*-2), 32.4, 32.1, 32.1, 32.1, 29.8, 29.8, 29.8, 29.6, 29.5, 29.4, 29.4, 29.2, 29.2, 27.5, 27.5 (26C, *C*-alkyl), 22.8 (2C, *C*-17), 22.5 (2C, *C*-5') 14.3 (2C, *C*-18), 14.0 (2C, *C*-6') ppm; HRMS-ESI (+), *m/z*: 899.70359 [2M+Na$^+$] calcd. for $C_{48}H_{92}N_8NaO_6^+$: 899.70320.

α -Azido-C16-ceramide **CerC16*α***



General procedure A gave **CerC16*α*** as colorless solid. Yield 94 %. R_f: 0.44 (CH_2Cl_2/MeOH 10:1). ^{1}H-NMR: (400 MHz, $CDCl_3$): δ = 7.01 (d, ^3J = 7.9 Hz, 2H; N*H*), 5.80 (dddd, ^3J = 15.4, 6.8, 6.8 ^4J = 1.2 Hz, 2H; *H*-5), 5.53 (dddd, ^3J = 15.4, 6.5, ^4J = 1.4, 1.4 Hz, 1H; *H*-4) respectively 5.52 (dddd, ^3J = 15.4, 6.5, ^4J = 1.4, 1.4 Hz, 1H; *H*-4), 4.37-4.27 (m, 2H; H-3), 4.01-3.97 (m, 4H, H-1a, *H*-2'), 3.89 (ddd, ^3J = 7.9, 7.9, 3.6, Hz; 1H, *H*-2) respectively 3.88 (ddd, ^3J = 7.8, 7.8, 3.6, Hz; 1H, *H*-2), 3.76-3.66 (m, 2H, *H*-1), 2.70-2.54 (m, 2H, *C*-1-O*H*), 2.54-2.43 (m, 2H, *C*-2-O*H*), 2.10-2.01 (m, 4H, *H*-6), 1.97-1.88 (m, 2H, *H*-3‘a), 1.83 (dddd, ^3J = 14.5, 7.3, 7.3, 7.3 Hz; 2H, *H*-3'b), 1.47-1.19 (m, 92Hs, *H*-alkyl), 0.88 (t, 6.9 Hz, 12H, *H*-16', *H*-18) ppm; ^{13}*C*-NMR: (100 MHz, $CDCl_3$): 170,1 (2C, *C*-1'), 134,9 (2C, *C*-5), 128,7 (2C, *C*-4), 74,6 (1C, *C*-3), 74,5 (1C, *C*-3), 64,6 (2C, *C*-2'), 62.4 (1C, *C*-1), 62.3 (1 C, *C*-1), 54.6 (2C, *C*-2), 32.4, 32.4, 32.1, 29.8, 29.8, 29.8, 29.7, 29.7, 29.6, 29.6, 29.5, 29.5, 29.4, 29.4, 29.2, 29.2, 25.5, 25.5 (46C, *C*-alkyl), 22.8 (4C, *C*-17/*C*-15'), 14.3 (4C, *C*-18/*C*-16') ppm; HRMS-ESI (+), *m/z*: 601.50271 [M+Na$^+$] calcd. for $C_{34}H_{66}N_4NaO_3^+$: 601.50271.

ω-Azidohexanoic acid **1**

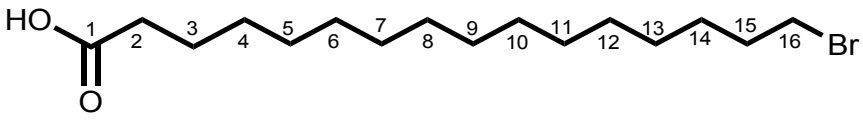


Tf_2O (2.00 eq.) was added dropwise to a mixture of NaN_3 (10.0 eq.) in water (0.25 ml per mmol NaN_3) and CH_2Cl_2 (0.5 ml per mmol NaN_3) at 0 °C. The mixture was stirred vigorously for 2 h

and the organic layer separated. The aqueous layer was extracted two times with CH_2Cl_2 (0.20 ml per mmol NaN_3). The organic layers were combined and used immediately in the next reaction without any further purification. (Handle this solution with caution due its explosive and toxic nature, do not concentrate!)

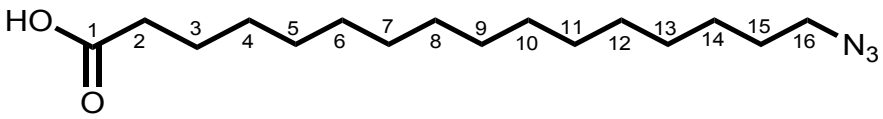
The freshly prepared solution of TfN_3 was added dropwise to a mixture of *ω*-aminohexanoic acid (1.00 eq.) acid, K_2CO_3 (2.00 eq.), $CuSO_4$ hexahydrate (0.01 eq.), MeOH (2.50 ml per mmol *ω*-aminohexanoic) and H_2O (1.00 ml per mmol *ω*-aminohexanoic acid) at 0 °C. The mixture was stirred at rt for 16 h. Water was added (2.50 mL per mmol *ω*-aminohexanoic acid) and the mixture was washed with CH_2Cl_2 (five times with 5.00 mL per mmol *ω*-aminohexanoic acid each). The aqueous phase were acidified to pH = 2 with 1 M HCl and extracted with CH_2Cl_2 (five times with 5.00 ml per mmol *ω*-aminohexanoic acid each). The organic layers of the extraction were combined, the solvents removed under reduced pressure to give **1** as a colorless liquid. Yield 99 %. R_f: 0.45 (EE). ^1H-NMR: (400 MHz, $CDCl_3$): δ = 3.28 (t, ^3J = 6.8 Hz, 2H; H-6), 2.38 (t, ^3J = 7.4 Hz, 2H; H-2), 1.71-1.59 (m, 4H, *H*-3/5) 1.47-1.39 (m, 2H, H-4) ppm. ^{13}C-NMR: (100 MHz, $CDCl_3$): δ = 179.9 (*C*-1), 51.3 (*C*-6), 33.9 (*C*-2), 28.7 (*C*-5), 26.3 (*C*-4), 24.3 (*C*-3) ppm. HRMS-ESI (+), m/z: 180.07537 [M+Na^+] calcd. for $C_6H_{11}N_3NaO_2^+$: 180.07544.

ω-Bromohexadecanoic acid **2**



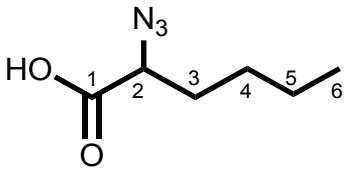
ω-Hydroxyoctadecanoic Acid (1.00 eq.) was solved in a mixture of glacial acetic acid (5.00 mL per mmol *ω*-hydroxyoctadecanoic acid) and 48 % HBr aq. (5.00 ml per mmol *ω*-hydroxyoctadecanoic acid). The mixture was refluxed for 24 h. The mixture was cooled to 0 °C and filtrated. The residue was recrystallized two times from cyclohexane to give **3** as a colorless solid. Yield: 94 %. R_f: 0.53 (cyclohexane/EE 1:1). ^1H-NMR: (400 MHz, $CDCl_3$): δ = 33.41 (t, ^3J = 6.9 Hz, 2H; *H*-16), 2.35 (t, ^3J = 7.5 Hz, 2H; *H*-2), 1.84 (tt, ^3J = 7.3, 7.3 Hz, 2H; *H*-15), 1.62 (tt, ^3J = 7.4, 7.4 Hz, 2H; *H*-3), 1.46-1.38 (m, 2H, *H*-4), 1.38-1.20 (m, 20H, *H*-5 to *H*-14) ppm. ^{13}C-NMR: (100 MHz, $CDCl_3$): δ = 179.6 (*C*-1), 34.3 (*C*-16), 34.1 (*C*-2), 33.0 (*C*-15), 29.8, 29.8, 29.7, 29.6, 29.6, 29.4, 28.9 (10C, *C*-alkyl) 28.6 (*C*-4), 24.8 (*C*-3)ppm.

ω-Azidohexadecanoic acid **3**



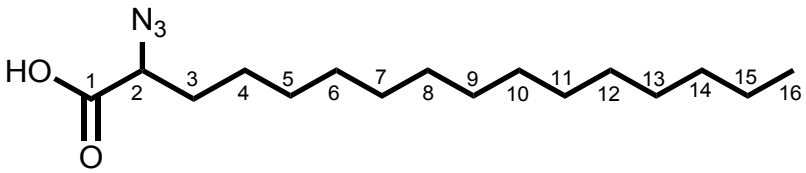
General procedure B and column chromatography (cyclohexane/ethyl acetate 4:1) gave **4** as colorless solid. Yield: 99 %. R_f: 0.27 (cyclohexane/ethyl acetate 2:1). ^{1}*H*-NMR: (400 MHz, $CDCl_3$): δ = = 3.25 (t, ^3J = 7.0 Hz, 2H; *H*-16), 2.35 (t, ^3J = 7.5 Hz, 2H; *H*-2), 1.62 (tt, ^3J = 7.0, 7.0 Hz, 2H; *H*-15), 1.58 (tt, ^3J = 7.1, 7.1 Hz, 2H; *H*-3), 1.41-1.20 (m, 22H, *H*-4 to *H*-14) ppm. ^{13}*C*-NMR: (100 MHz, $CDCl_3$): δ = 179.9 (*C*-1), 51.6 (*C*-16), 34.1 (*C*-2), 29.8, 29.7, 29.7, 29.6, 29.6, 29.4, 29.3, 29.2, 29.0 (11C, *C*-alkyl), 26.9 (*C*-4), 24.8 (*C*-3) ppm. HRMS-ESI (+), m/z: 298.24985 [M+H$^+$] calcd. for $C_{16}H_{32}N_3O_2^+$: 298.24890.

α-Azidohexanoic acid **4**



General procedure B and column chromatography (cyclohexane/ethyl acetate 4:1) gave **2** as colorless liquid. Yield: 85 %. R_f: 0.66 (ethyl acetate/isopropanol/H_2O 6:3:1). ^{1}H-NMR: (400 MHz, $CDCl_3$): δ = 11.96 (s, 1H, COO*H*), 4.23 (t, ^3J = 7.8, 7.0 Hz, 1H; *H*-2), 2.13-1.94 (m, 2H; *H*-3), 1.53-1.28 (m, 4H; *H*-4/5), 0.92 (t, ^3J = 7.1 Hz, 3H, *H*-3) ppm. ^{13}*C*-NMR: (100 MHz, $CDCl_3$): δ =176.6 (*C*-1), 45.5 (*C*-2), 34.4 (*C*-3), 29.4 (*C*-4), 22.1 (*C*-5), 13.9 (*C*-6) ppm.

α-Azidohexadecanoic acid **5**

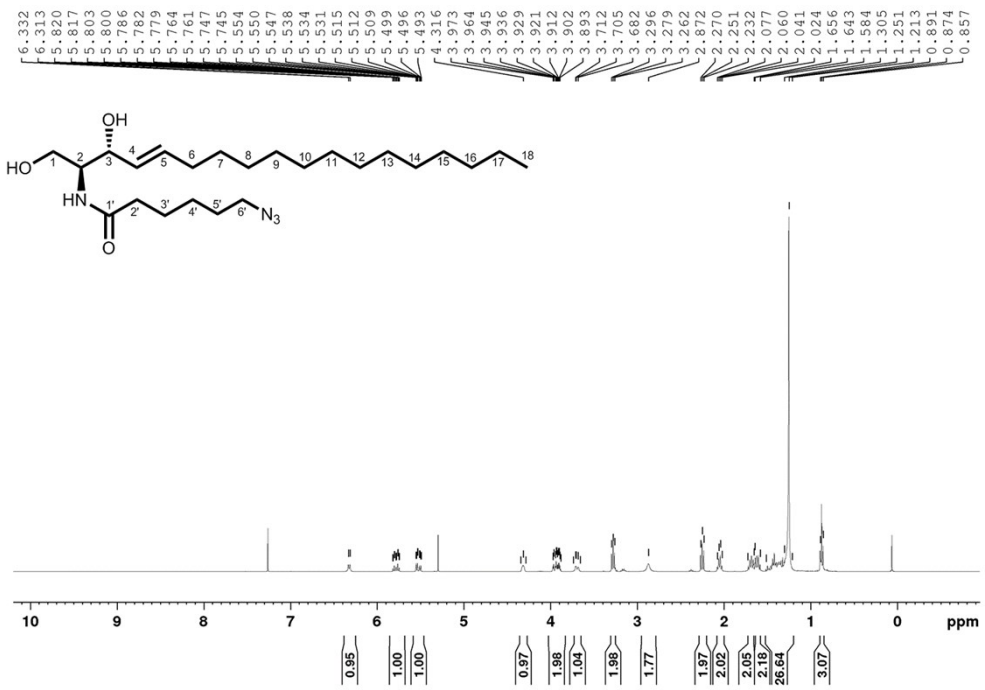


General procedure B and column chromatography (cyclohexane/ethyl acetate 4:1) gave **4** as colorless solid. Yield: 95 %. R_f: 0.32 (cyclohexane/ethyl acetate 2:1). ^{1}H-NMR: (400 MHz, $CDCl_3$/MeOD 4:1): = 3.89 (dd, ^3J = 5.2, 8.4, 1H, *H*-2), 1.94-1.76 (m, 2H, *H*-3), 1.54–1.19 (m, 24H,

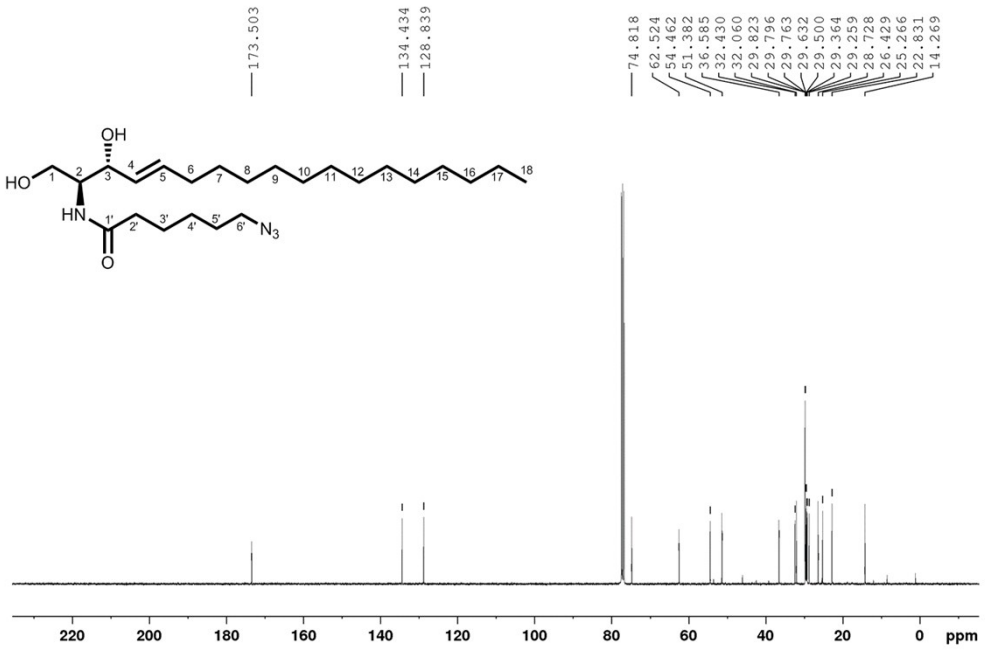
H-4 to *H*-15), 0.88 (t, ^{3}J = 6.9 Hz, 3H, *H*-16) ppm. ^{13}C-NMR: (100 MHz, MeOD): δ = 176.3 (*C*-1), 61.8 (*C*-2), 32.1, 31.4, 29.8, 29.8, 29.8, 29.8, 29.7, 29.6, 29.5, 29.5, 29.1, 25.8, 22.8 (13C, *C*-alkyl), 14.3 (*C*-18) ppm. HRMS-ESI (+), m/z: 320.23115 [M+Na^{+}] calcd. for $C_{16}H_{31}N_3NaO_2^{+}$: 320.23085.

NMR-Spetcra

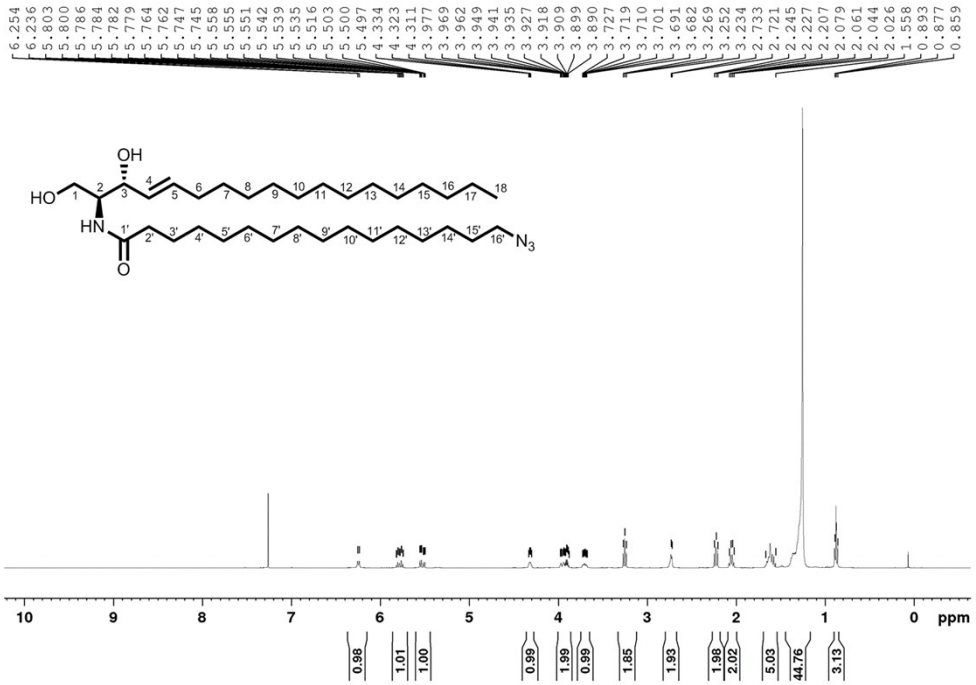
^{1}H-NMR (400 MHz, $CDCl_3$) of *ω*-Azido-C6-ceramide **CerC6*ω***



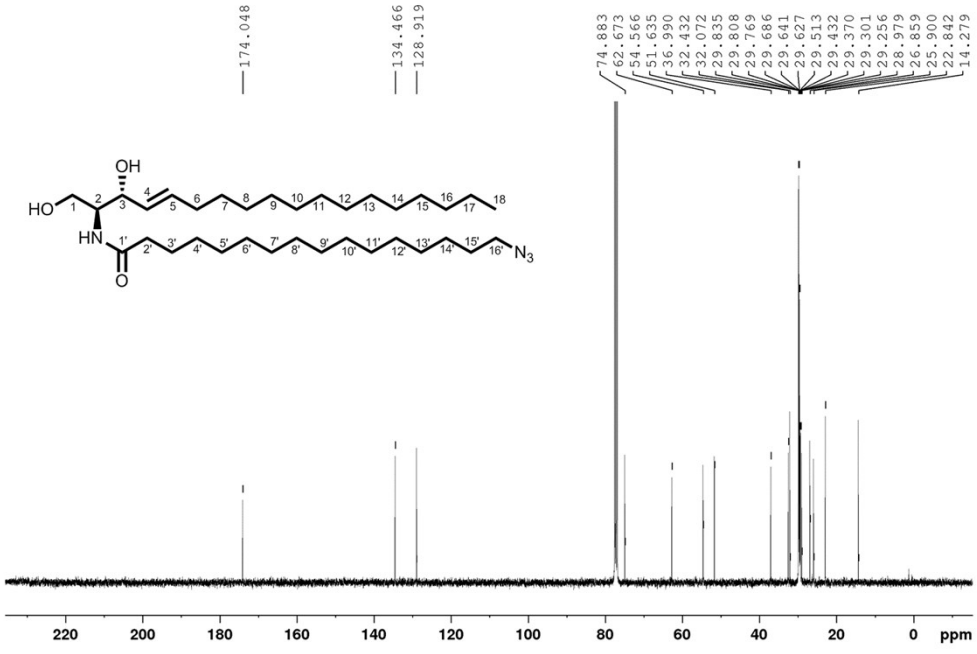
^{13}C-NMR (100 MHz, $CDCl_3$) of *ω*-Azido-C6-ceramide **CerC6*ω***



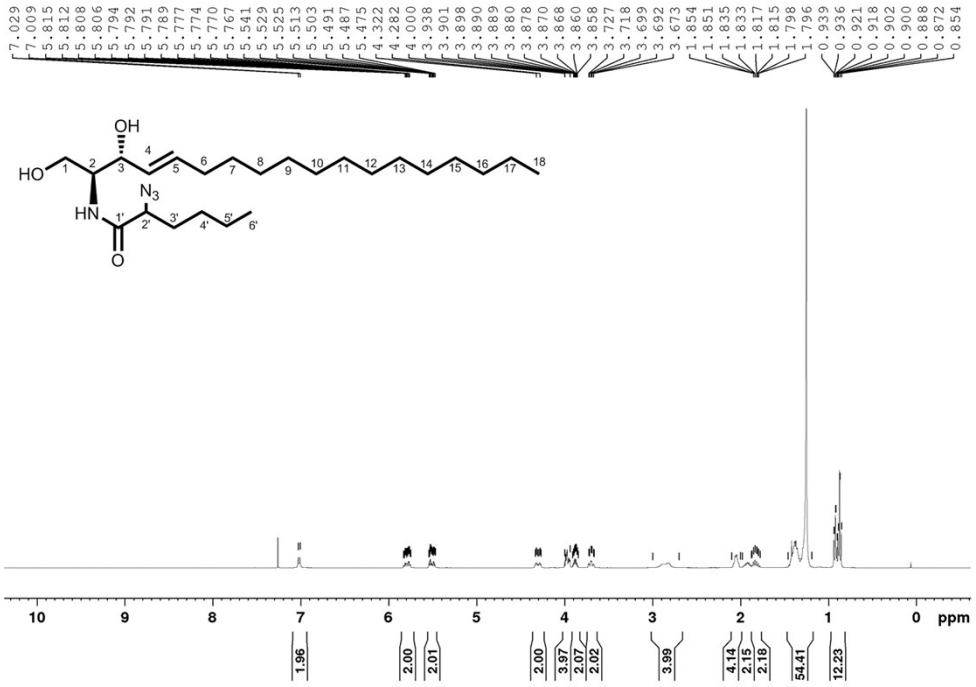
^{1}H-NMR (400 MHz, $CDCl_3$) of *ω*-Azido-C16-ceramide **CerC16*ω***



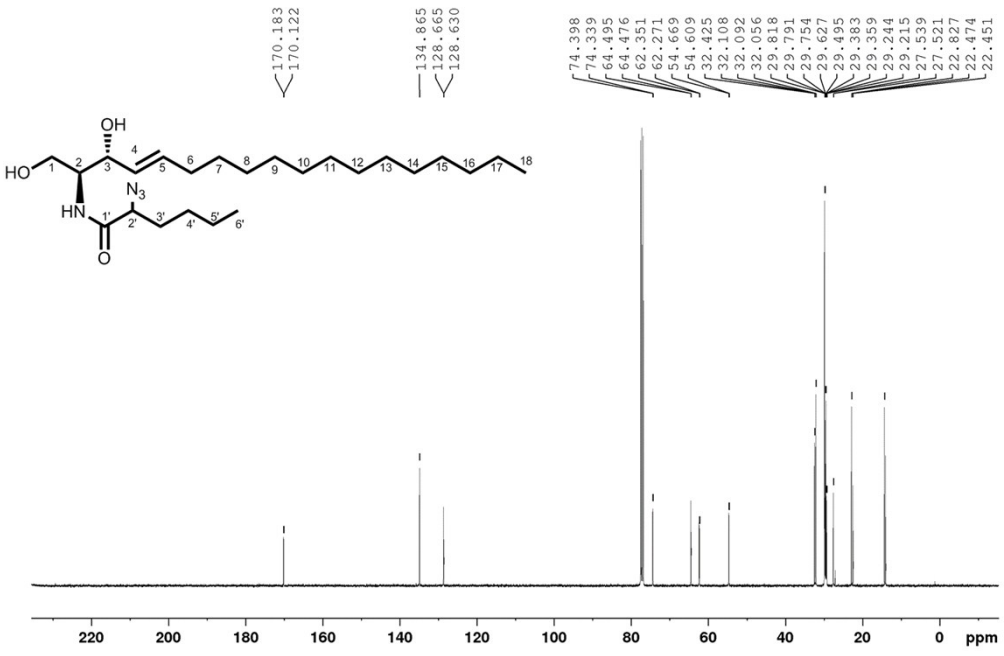
^{13}C-NMR (100 MHz, $CDCl_3$) of *ω*-Azido-C16-ceramide **CerC16*ω***



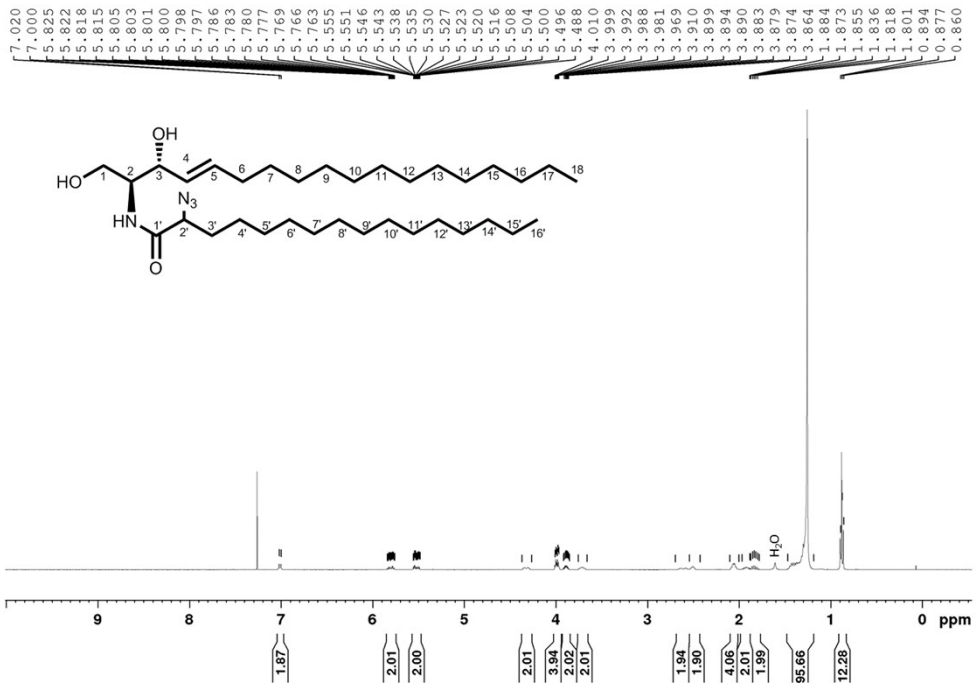
^{1}H-NMR (400 MHz, $CDCl_3$) of *ω*-Azidohexadecanoic acid **CerC16*α***



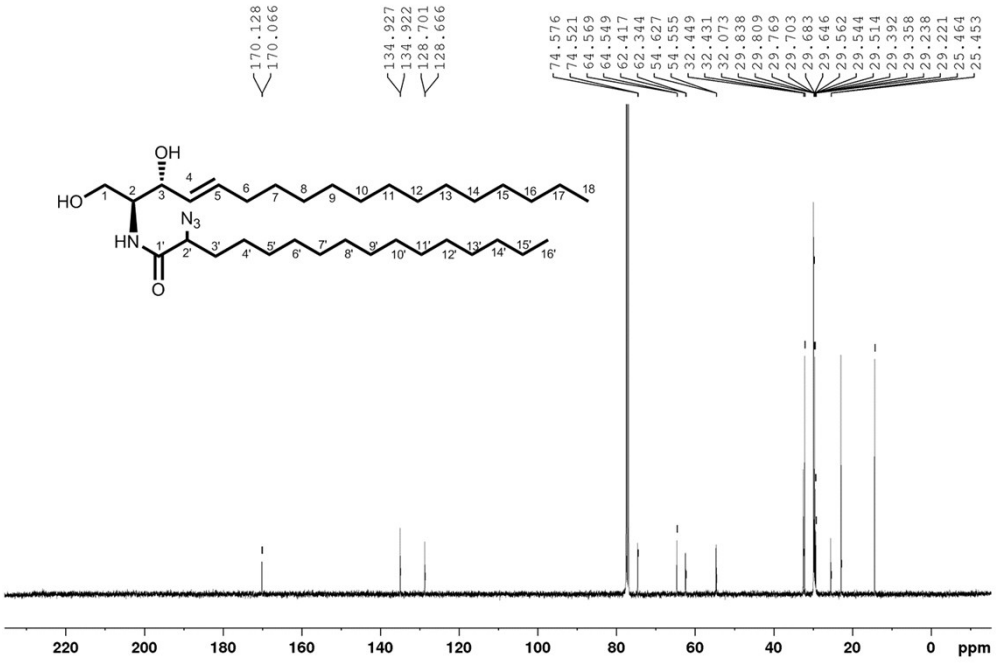
^{13}C-NMR (100 MHz, $CDCl_3$) of *ω*-Azidohexadecanoic acid **CerC16*α***



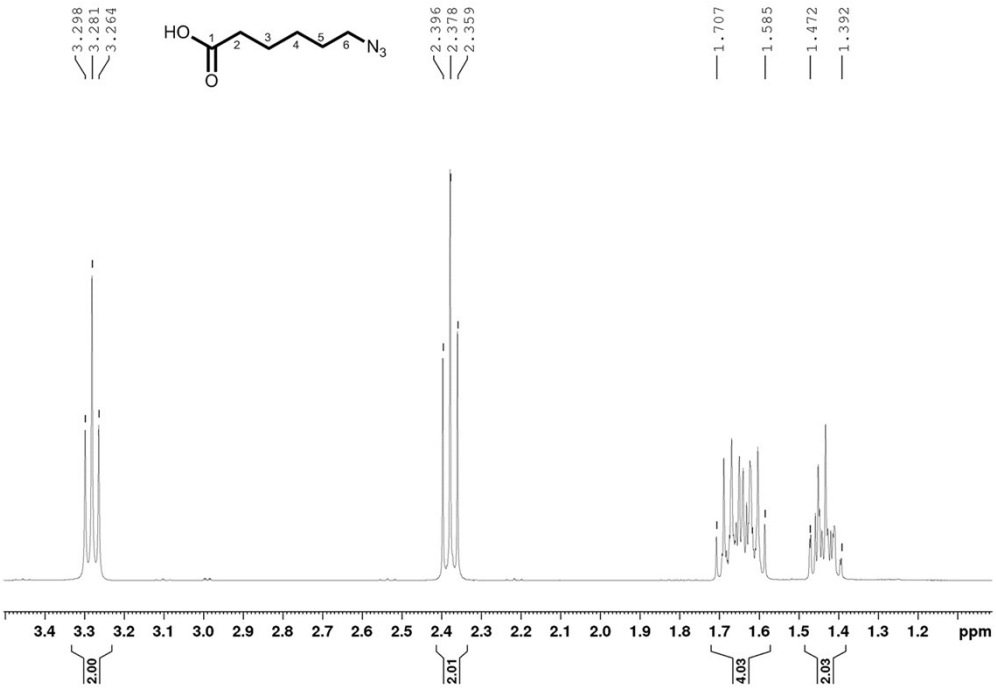
^{1}H-NMR (400 MHz, $CDCl_3$) of ω-Azidohexadecanoic acid **CerC16*α***



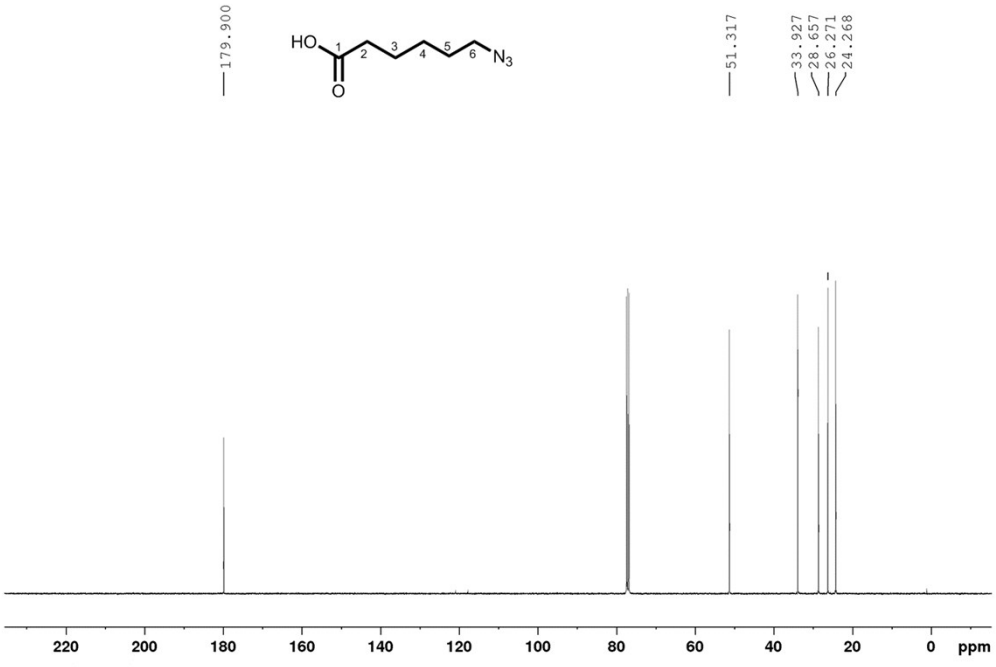
^{13}C-NMR (100 MHz, $CDCl_3$) of ω-Azidohexadecanoic acid **CerC16*α***



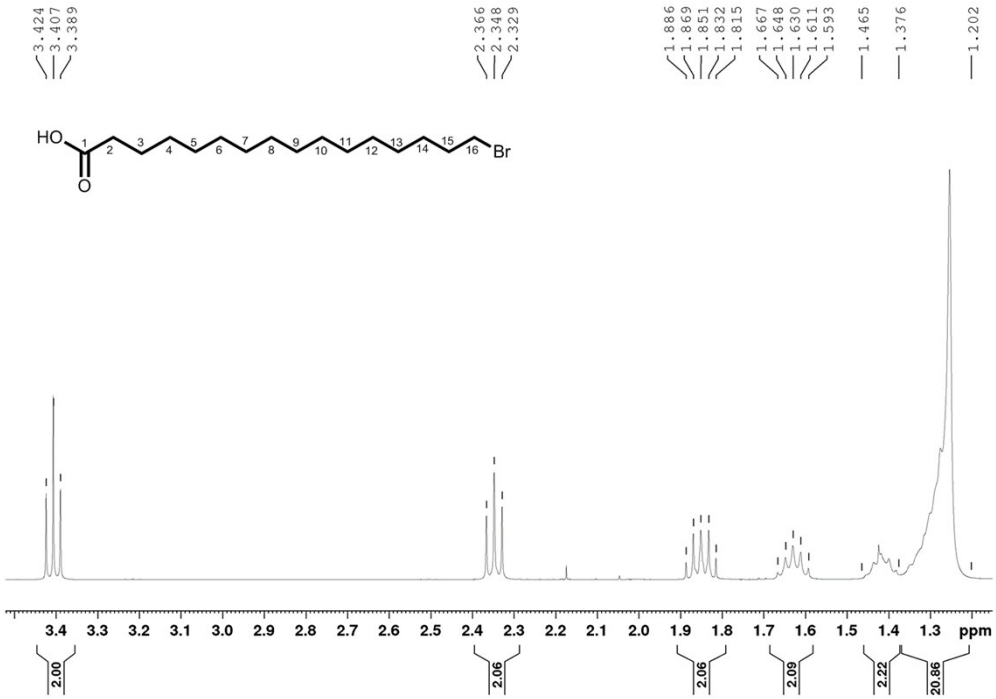
^{1}H-NMR (400 MHz, $CDCl_3$) of *ω*-Azidohexanoic acid **1**



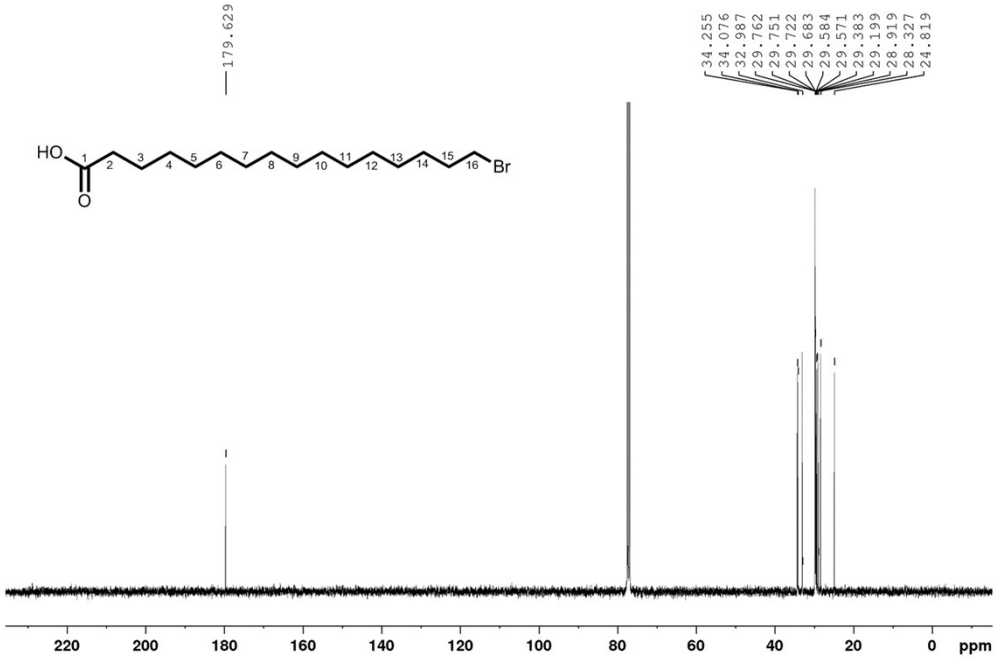
^{13}C-NMR (100 MHz, $CDCl_3$) of *ω*-Azidohexanoic acid **1**



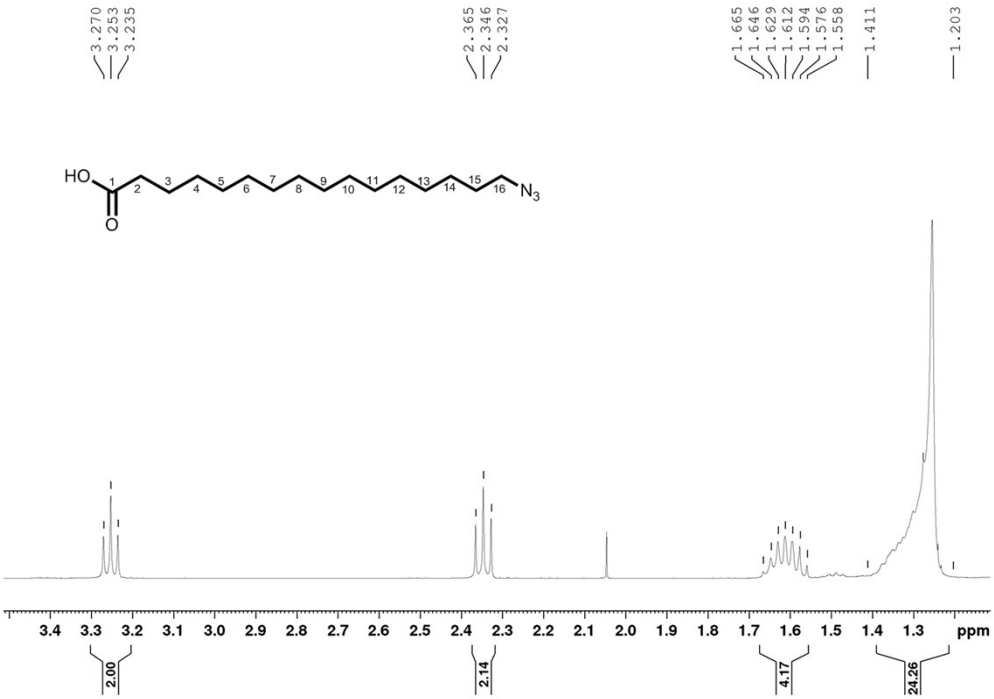
^{1}H-NMR (400 MHz, $CDCl_3$) of ω-Bromohexadecanoic acid **2**



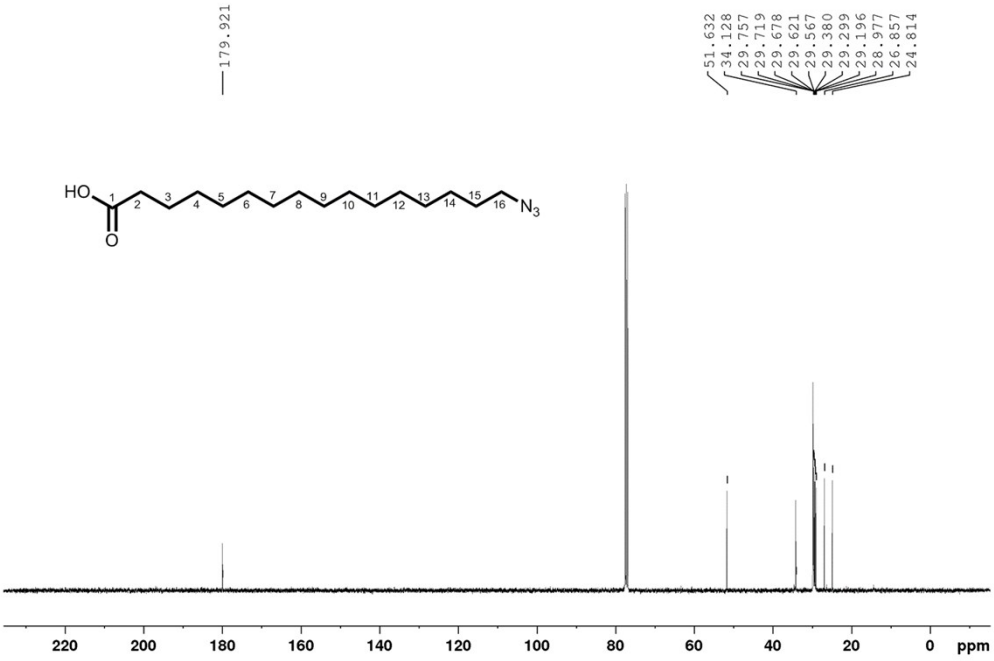
^{13}C-NMR (100 MHz, $CDCl_3$) of ω-Bromohexadecanoic acid **2**



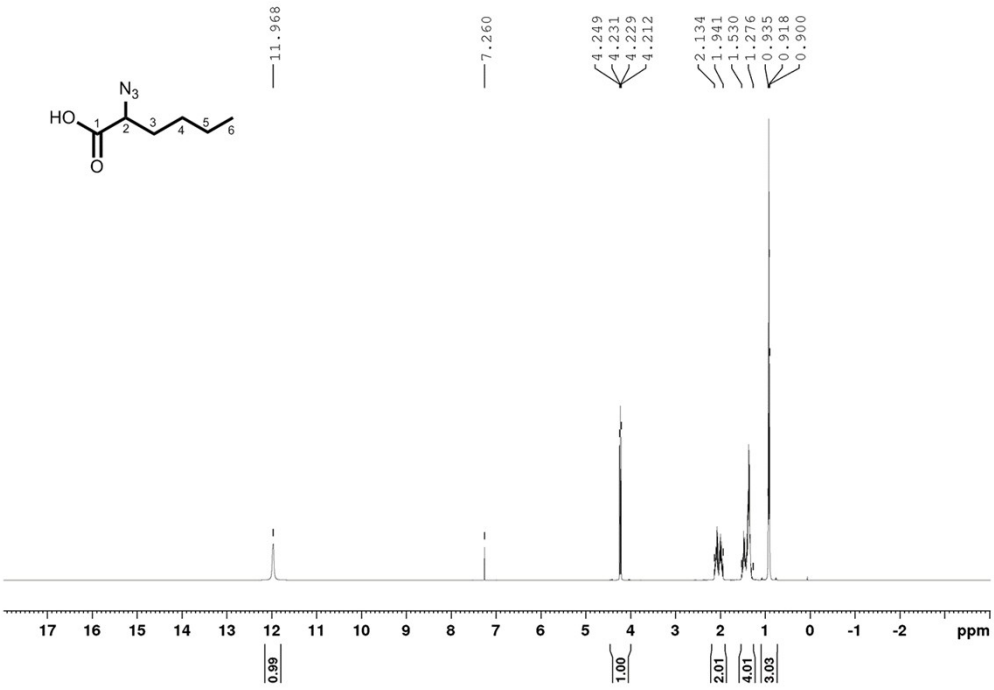
^{1}H-NMR (400 MHz, $CDCl_3$) of *ω*-Azidohexadecanoic acid **3**



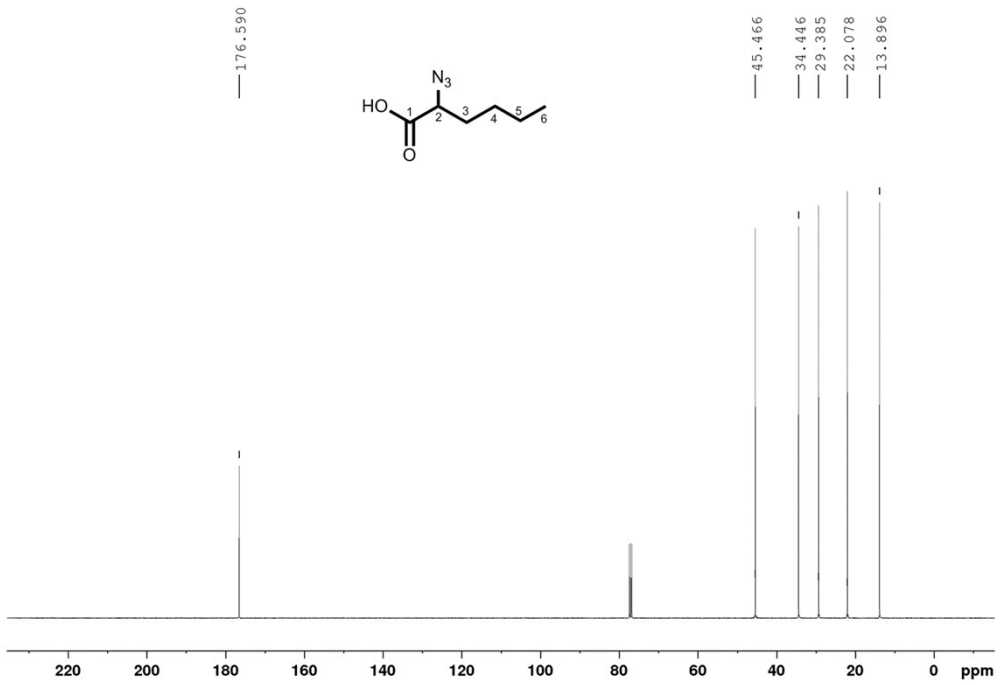
^{13}C-NMR (100 MHz, $CDCl_3$) of *ω*-Azidohexadecanoic acid **3**



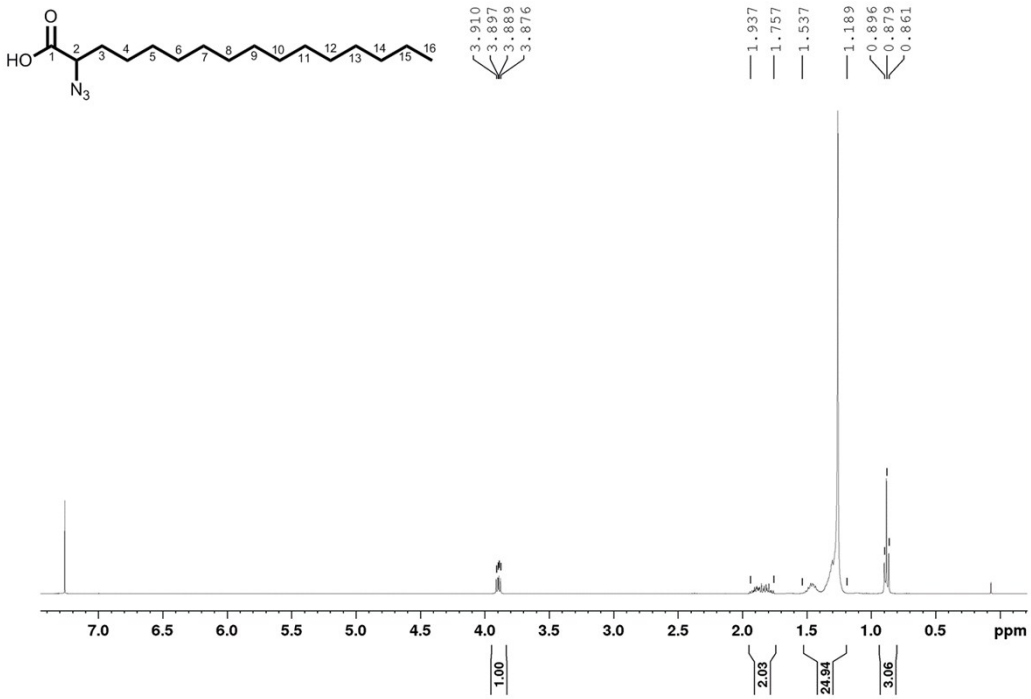
^{1}H-NMR (400 MHz, $CDCl_3$) of *ω*-Azidohexadecanoic acid **4**



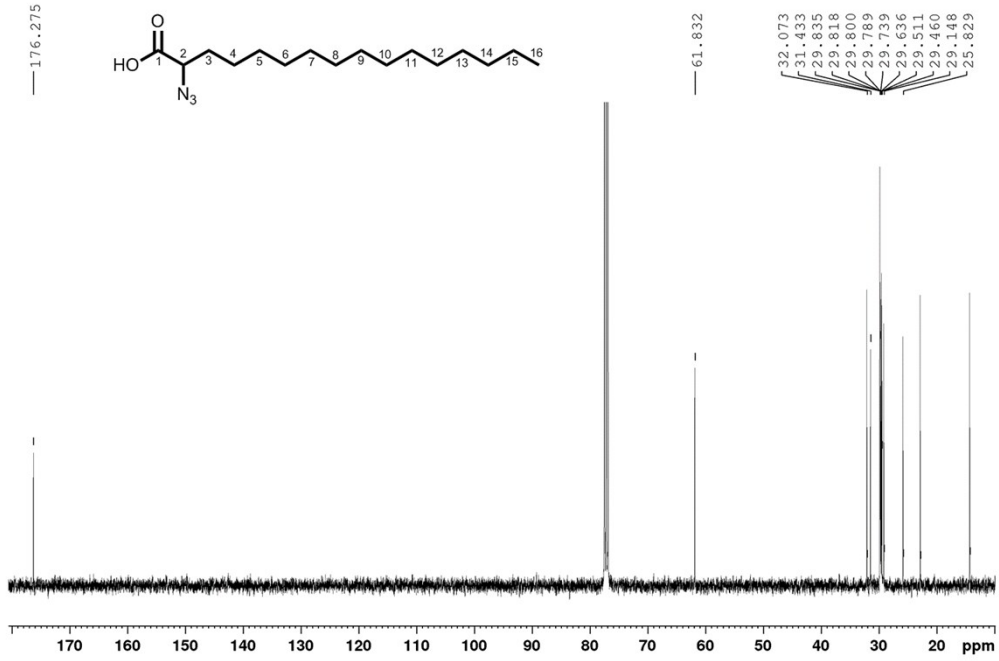
^{13}C-NMR (100 MHz, $CDCl_3$) of *ω*-Azidohexadecanoic acid **4**



^{1}H-NMR (400 MHz, $CDCl_3$) of *ω*-Azidohexadecanoic acid **5**

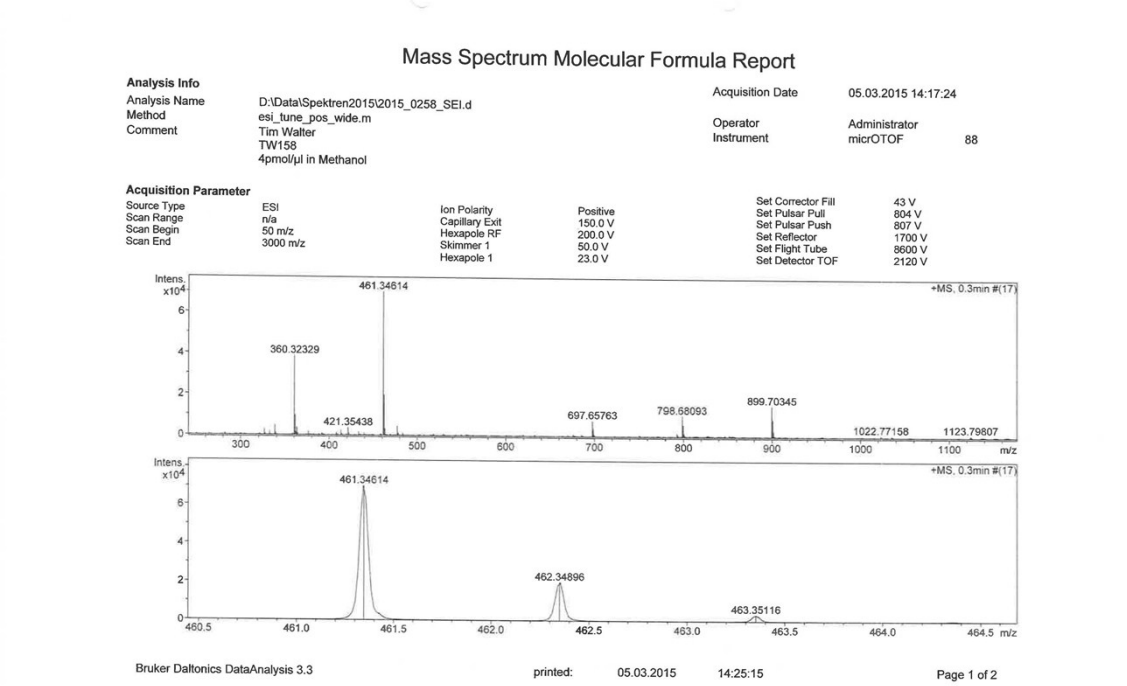


^{13}C-NMR (100 MHz, $CDCl_3$) of *ω*-Azidohexadecanoic acid **5**

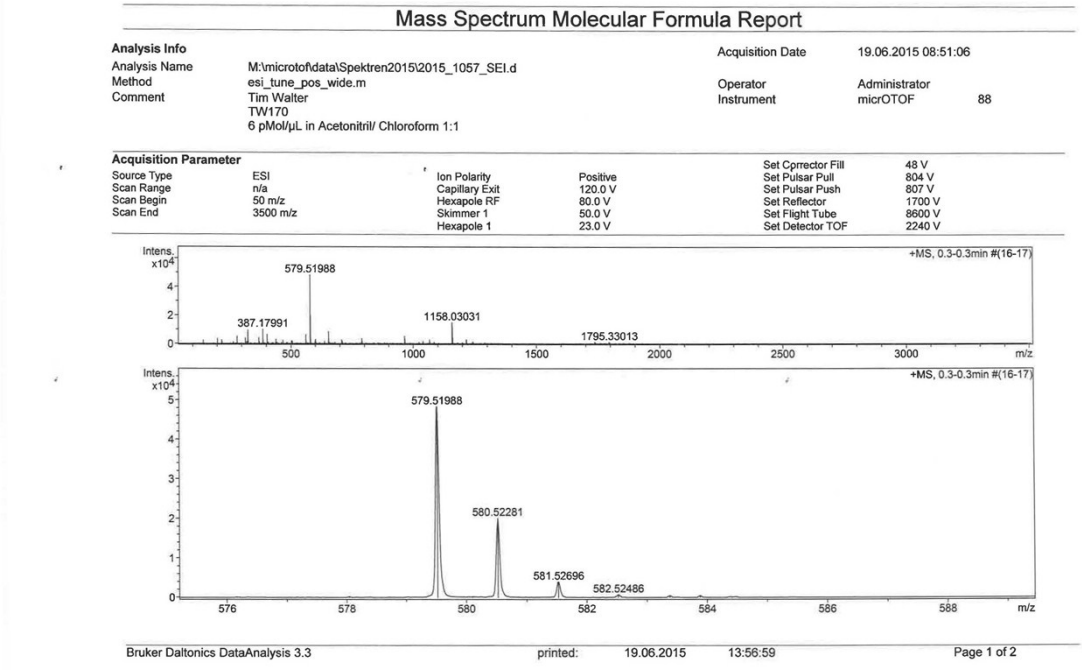


Mass Spectrometry

ω-Azido-C6-ceramide **CerC6*ω***



ω-Azido-C16-ceramide **CerC16*ω***



α-Azido-C16-ceramide **CerC6*α***

Bruker Daltonics DataAnalysis 3.3

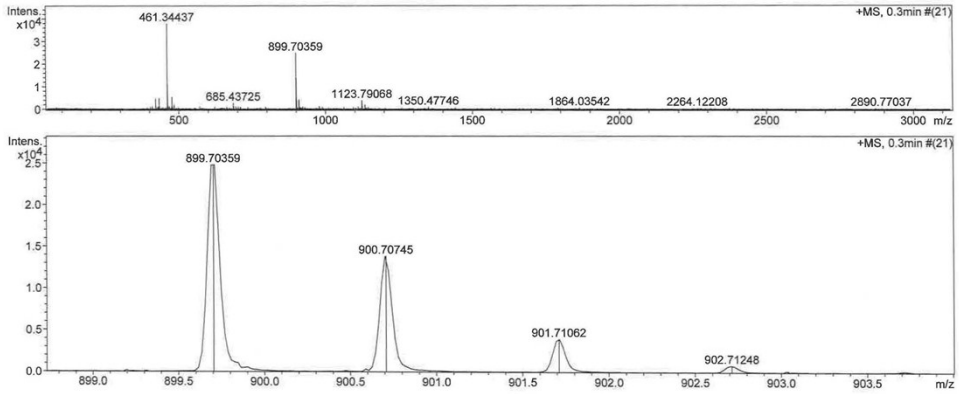
Mass Spectrum Molecular Formula Report

Analysis Info

Analysis Name	D:\Data\Spektren2016\2016_0845_SEI_2.d	Acquisition Date	18.04.2016 14:00:10
Method	esi_tune_pos_wide.m		
Comment	Tim Walter TW191 4pmol/µL in Acetonitril/Chloroform 1:1	Operator Instrument	Administrator micrOTOF 88

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Corrector Fill	48 V
Scan Range	n/a	Capillary Exit	180.0 V	Set Pulsar Pull	804 V
Scan Begin	50 m/z	Hexapole RF	600.0 V	Set Pulsar Push	807 V
Scan End	3000 m/z	Skimmer 1	50.0 V	Set Reflector	1700 V
		Hexapole 1	23.0 V	Set Flight Tube	8600 V
				Set Detector TOF	2200 V



printed: 18.04.2016 14:07:13 Page 1 of 2

ω-Azido-C16-ceramide **CerC16*α***

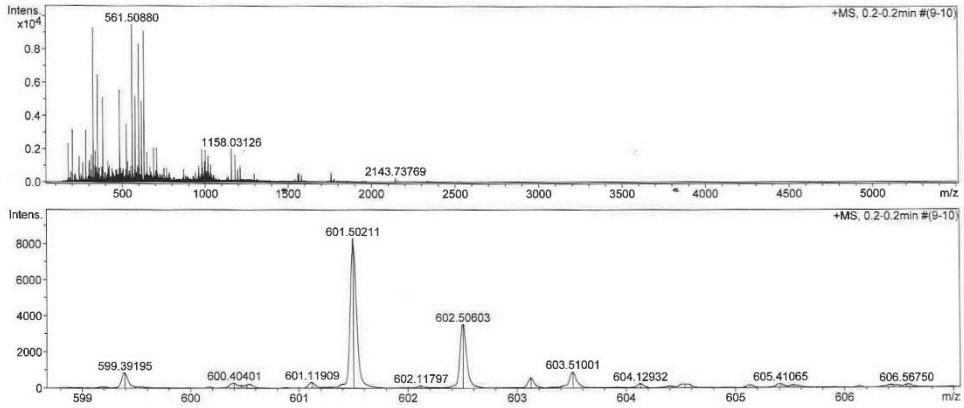
Mass Spectrum Molecular Formula Report

Analysis Info

Analysis Name	D:\Data\Spektren2015\2015_1541_SEI.d	Acquisition Date	06.08.2015 11:14:45
Method	esi_tune_pos_wide.m		
Comment	Tim Walter TW172 6 pMol/µL in Chloroform/ Acetonitril 1:1	Operator Instrument	Administrator micrOTOF 88

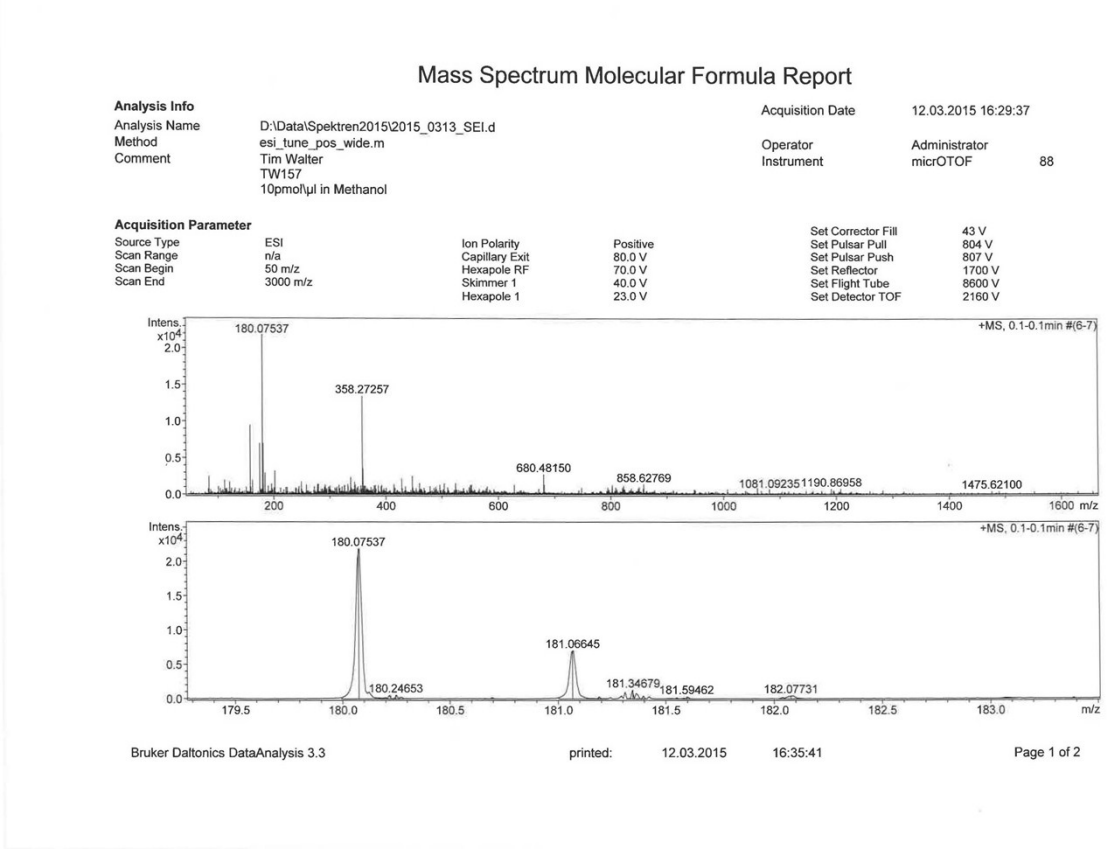
Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Corrector Fill	48 V
Scan Range	n/a	Capillary Exit	150.0 V	Set Pulsar Pull	804 V
Scan Begin	50 m/z	Hexapole RF	180.0 V	Set Pulsar Push	807 V
Scan End	5500 m/z	Skimmer 1	50.0 V	Set Reflector	1700 V
		Hexapole 1	23.0 V	Set Flight Tube	8600 V
				Set Detector TOF	2150 V



Bruker Daltonics DataAnalysis 3.3 printed: 06.08.2015 12:08:09 Page 1 of 2

ω-Azidohexanoic acid **1**



Mass Spectrum Molecular Formula Report

Analysis Info

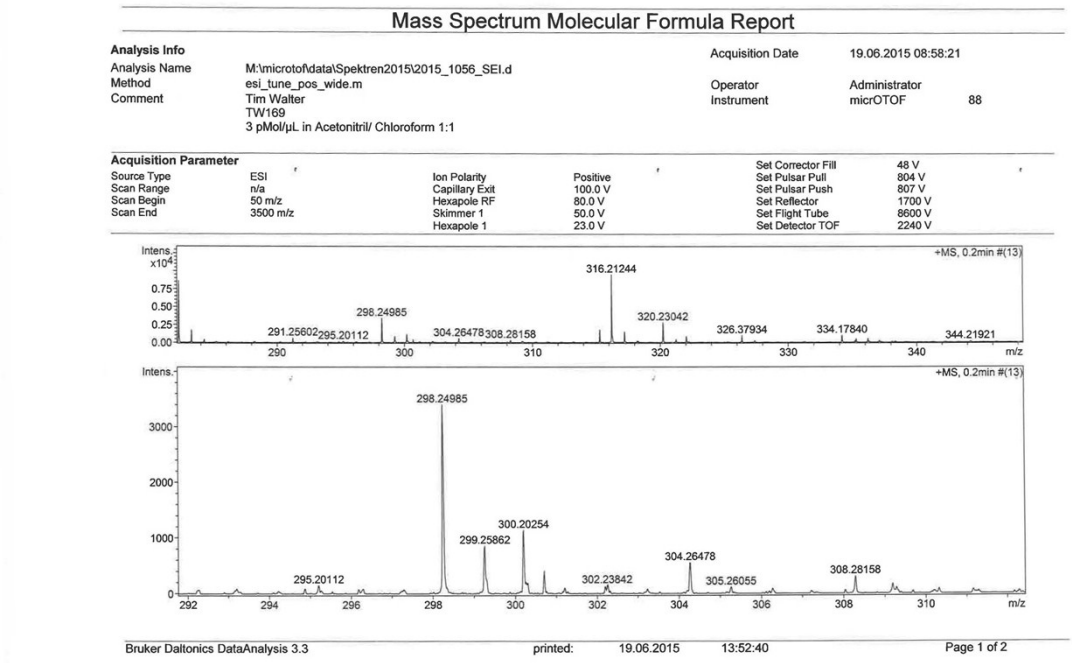
Analysis Name	D:\Data\Spektren2015\2015_0313_SEI.d	Acquisition Date	12.03.2015 16:29:37
Method	esi_tune_pos_wide.m	Operator	Administrator
Comment	Tim Walter TW157 10pmol\µl in Methanol	Instrument	micrOTOF 88

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Corrector Fill	43 V
Scan Range	n/a	Capillary Exit	80.0 V	Set Pulsar Pull	804 V
Scan Begin	50 m/z	Hexapole RF	70.0 V	Set Pulsar Push	807 V
Scan End	3000 m/z	Skimmer 1	40.0 V	Set Reflector	1700 V
		Hexapole 1	23.0 V	Set Flight Tube	8600 V
				Set Detector TOF	2160 V

Bruker Daltonics DataAnalysis 3.3 printed: 12.03.2015 16:35:41 Page 1 of 2

ω-Azidohexadecanoic acid **3**



Mass Spectrum Molecular Formula Report

Analysis Info

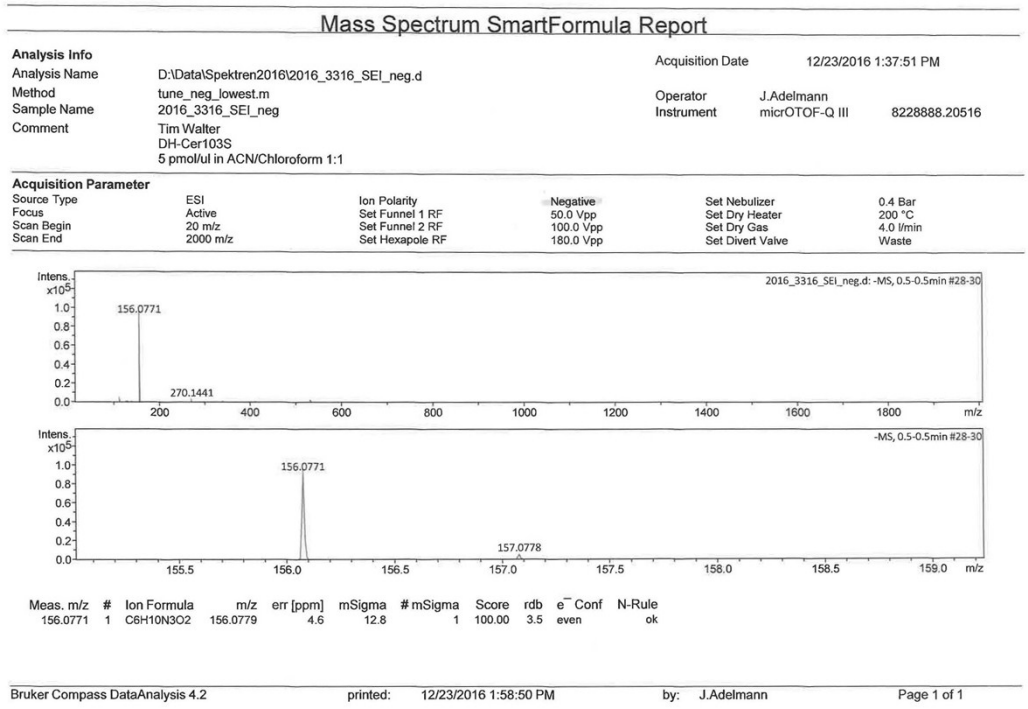
Analysis Name	M:\microtof\data\Spektren2015\2015_1056_SEI.d	Acquisition Date	19.06.2015 08:58:21
Method	esi_tune_pos_wide.m	Operator	Administrator
Comment	Tim Walter TW169 3 pMol/µL in Acetonitril/ Chloroform 1:1	Instrument	micrOTOF 88

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Corrector Fill	48 V
Scan Range	n/a	Capillary Exit	100.0 V	Set Pulsar Pull	804 V
Scan Begin	50 m/z	Hexapole RF	80.0 V	Set Pulsar Push	807 V
Scan End	3500 m/z	Skimmer 1	50.0 V	Set Reflector	1700 V
		Hexapole 1	23.0 V	Set Flight Tube	8600 V
				Set Detector TOF	2240 V

Bruker Daltonics DataAnalysis 3.3 printed: 19.06.2015 13:52:40 Page 1 of 2

α-Azidohexanoic acid **4**



Mass Spectrum SmartFormula Report

Analysis Info

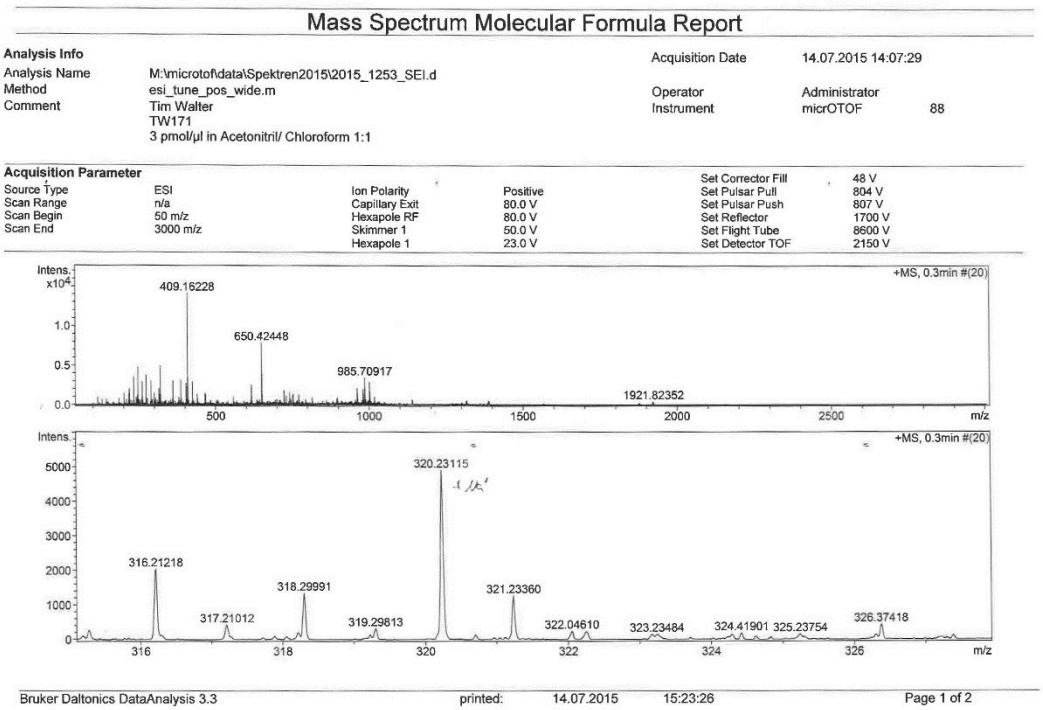
Analysis Name	D:\Data\Spektren2016\2016_3316_SEI_neg.d	Acquisition Date	12/23/2016 1:37:51 PM
Method	tune_neg_lowest.m	Operator	J.Adelmann
Sample Name	2016_3316_SEI_neg	Instrument	micrOTOF-Q III 8228888.20516
Comment	Tim Walter DH-Cer103S 5 pmol/ul in ACN/Chloroform 1:1		

Acquisition Parameter

Source Type	ESI	Ion Polarity	Negative	Set Nebulizer	0.4 Bar
Focus	Active	Set Funnel 1 RF	50.0 Vpp	Set Dry Heater	200 °C
Scan Begin	20 m/z	Set Funnel 2 RF	100.0 Vpp	Set Dry Gas	4.0 l/min
Scan End	2000 m/z	Set Hexapole RF	180.0 Vpp	Set Divert Valve	Waste

Meas. m/z	#	Ion Formula	m/z	err [ppm]	mSigma	# mSigma	Score	rdb	e⁻ Conf	N-Rule
156.0771	1	C6H10N3O2	156.0779	4.6	12.8	1	100.00	3.5	even	ok

α-Azidohexadecanoic acid **5**



Mass Spectrum Molecular Formula Report

Analysis Info

Analysis Name	M:\microtof\data\Spektren2015\2015_1253_SEI.d	Acquisition Date	14.07.2015 14:07:29
Method	esi_tune_pos_wide.m	Operator	Administrator
Comment	Tim Walter TW171 3 pmol/µl in Acetonitril/ Chloroform 1:1	Instrument	micrOTOF 88

Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Corrector Fill	48 V
Scan Range	n/a	Capillary Exit	80.0 V	Set Pulsar Pull	804 V
Scan Begin	50 m/z	Hexapole RF	80.0 V	Set Pulsar Push	807 V
Scan End	3000 m/z	Skimmer 1	50.0 V	Set Reflector	1700 V
		Hexapole 1	23.0 V	Set Flight Tube	8600 V
				Set Detector TOF	2150 V

References

1. M. Boyce and C. R. Bertozzi, *Nat. Methods*, 2011, **8**, 638-642.
2. J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J.-Y. Tinevez, D. J. White, V. Hartenstein, K. Eliceiri, P. Tomancak and A. Cardona, *Nat. Methods*, 2012, **9**, 676-682.
3. J. C. Vaughan, G. T. Dempsey, E. Sun and X. Zhuang, *J. Am. Chem. Soc.*, 2013, **135**, 1197-1200.
4. J. R. Lakowicz, *Principles of Fluorescence Spectroscopy*, Springer Science Business Media, New York, 2006.